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Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state

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The microtubule-associated protein tau is a major component of the paired helical filaments (PHFs) observed in Alzheimer's disease brains. The pathological tau is distinguished from normal tau by its state of phosphorylation, higher apparent M_r and reaction with certain antibodies. However, the protein kinase(s) have not been characterized so far. Here we describe a protein kinase from brain which specifically induces the Alzheimer-like state in tau protein. The 42 kDa protein belongs to the family of mitogen activated protein kinases (MAPKs) and is activated by tyrosine phosphorylation. It is capable of phosphorylating Ser-Pro and Thr-Pro motifs in tau protein ($\approx 14-16$ P_i per tau molecule). By contrast, other proline directed Ser/Thr kinases such as p34(cdc2) combined with cyclin A or B have only minor effects on tau phosphorylation. We propose that MAP kinase is abnormally active in Alzheimer brain tissue, or that the corresponding phosphatases are abnormally passive, due to a breakdown of the normal regulatory mechanisms.
Key words: Alzheimer's disease/microtubules/mitogen activated protein kinase/paired helical filaments/tau protein

Introduction

The brains of Alzheimer patients contain two characteristic types of protein deposits, the amyloid plaques and neurofibrillary tangles. Much of current Alzheimer research is aimed at determining the nature of these deposits and the factors that cause them. A prominent component of the tangles are the paired helical filaments, PHFs, which are largely made up of the microtubule associated protein tau (for reviews see Kosik, 1990; Goedert *et al.*, 1991). This raises two general questions. In what way is PHF tau different from normal tau? What causes the difference? There are several isoforms of tau (six in human brain) which arise from alternative splicing of one gene (Goedert *et al.*, 1988, 1989; Lee *et al.*, 1988; Himmler *et al.*, 1989). The main differences between normal and PHF tau are the following: (i) PFH tau is highly insoluble and assembles into the paired helical filaments (Crowther and Wischik, 1985; Anderton, 1988); (ii) PHF tau can be distinguished from normal tau by certain antibodies whose reaction is often phosphorylation dependent [hence the conclusion that PHF tau is 'abnormally' phosphorylated (Grundke-Iqbal *et al.*, 1986; Wood *et al.*, 1986; Brion *et al.*, 1991; Biernat *et al.*, 1992)]; (iii) PHF is retarded in SDS gels, suggesting a higher M_r value; this effect is also related to phosphorylation (Flament and Delacourte, 1989; Lee *et al.*, 1991).

We have recently found a kinase activity in brain extracts which transformed tau into a state resembling that of PHFs. This analysis was based on a combination of several criteria. We first showed that certain monoclonal antibodies recognized tau from PHFs, but not normal tau, recombinant tau, or PHF tau after dephosphorylation. However, each of these protein preparations regained PHF-like immune response when they were treated with the kinase activity. Secondly, we determined by tryptic digestion and phosphopeptide sequencing that the transition to the PHF state included the phosphorylation of serines 46, 199, 202, 235, 396, 404 and 422, all of which are followed by prolines. Thirdly, we showed that the response to PHF-specific antibodies was linked to phosphorylation, and we determined several epitopes. For example, antibody AT8 depends on the phosphorylation of S199 and S202, antibodies SMI31 and SMI34 depend on the phosphorylation of S396 and S404. Antibody SMI33 requires an unphosphorylated S235, and TAU1 unphosphorylated S199 and S202, complementary to AT8 (in these cases the response is lost in the PHF state). Finally, we have verified by directed mutagenesis that the phosphorylatable serines were indeed involved in the transition of tau to the Alzheimer state, and that this transition generated a gel shift with each tau isoform (Biernat *et al.*, 1992; Lichtenberg-Kraag *et al.*, 1992; and unpublished data).

These observations strongly suggested that the kinase(s) were of the 'proline directed' type (for review see Pearson and Kemp, 1991; Hunter, 1991). Several of these kinases have recently been investigated, especially in the context of the cell cycle and activation by mitogens and growth factors. Examples are the kinases of the p34(cdc2) class which are complexed with cyclins A or B as regulatory subunits; these kinases phosphorylate histone H1 and other substrates and are normally turned off by phosphorylation at Tyr and other residues (reviews by Nurse, 1990; Hall and Vulliet, 1991). The opposite is true for the kinases of the MAPK type (Ray and Sturgill, 1988), also known as ERKs (Boulton *et al.*, 1991) or MBP kinases (Ahn *et al.*, 1990). They are part of a phosphorylation cascade in response to extracellular stimuli; one of their preferred substrates *in vitro* is MAP2 (for reviews see Cobb *et al.*, 1991; Sturgill and Wu, 1991; Thomas, 1992). We tested the effects of several proline directed kinases on tau to see if they induced Alzheimer-like characteristics similar to the kinase activity of the brain extract. In this study we have investigated the effects of cyclin dependent kinases and MAP kinases from several sources, we show that the Alzheimer characteristics of tau

are induced by a p42 MAP kinase, and we describe the purification of this kinase from brain.

Results

Cyclin dependent kinases

In searching for a proline directed Ser/Thr kinase that transforms tau into the Alzheimer state we first tested the p34−cycB complex, prepared from starfish oocytes according to Labbé *et al.* (1991) (Figure 1a). This kinase is involved in the transition to mitosis and phosphorylates Ser-Pro or Thr-Pro motifs in histone H1. It incorporates only ~0.1 P_i into the recombinant human tau, even with incubation times up to 24 h; there is no M_r shift with recombinant tau or normal brain tau (lanes 1−4), and the protein is not recognized by antibodies specific for phosphorylated sites in PHF tau (data not shown). For comparison, the kinase activity in brain extract incorporates up to 6−7 P_i, it causes a marked M_r shift, and it induces the Alzheimer-like response with several antibodies (Biernat *et al.*, 1992; Lichtenberg-Kraag *et al.*, 1992). These results made it unlikely that p34−cycB was the kinase responsible for the transformation of tau.

As an alternative we tried the starfish oocyte p34−cycA complex (Figure 1b). This kinase was more effective than p34−cycB, incorporating ≈2−3 P_i into recombinant tau in 24 h. However, this is still much lower than the phosphorylation level achieved with the brain extract, and in addition there was no Alzheimer-like antibody response nor an M_r shift. Thus, p34−cycA was not considered a good candidate for an 'Alzheimer kinase' either.

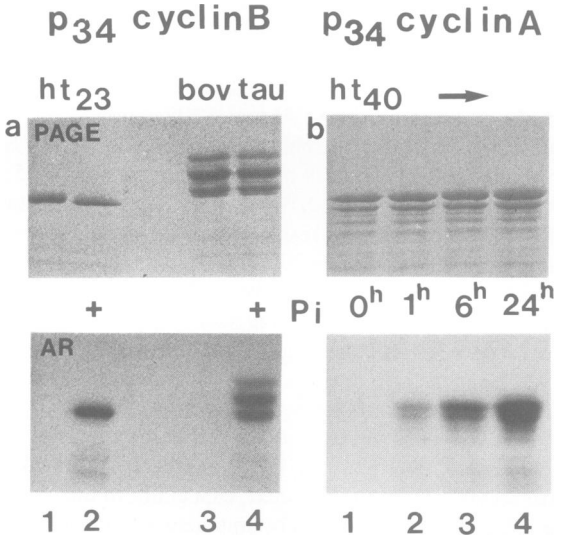

Fig. 1. Phosphorylation of bacterially expressed human isoforms and bovine brain tau with p34−cycB (**a**) or p34−cycA (**b**) (purified from starfish oocytes according to Labbé *et al.*, 1991). (a) Top, SDS gradient gel (4−20%) of htau23 (the smallest human isoform) without and with phosphorylation by p34−cycB (lanes 1 and 2), and of tau prepared from bovine brain, without and with phosphorylation (lanes 3 and 4). Note that there is no shift in M_r. Bottom, autoradiogram of the SDS gel, showing that some phosphate is incorporated into htau23 (lane 2) and bovine tau (lane 4). Quantitation of the band shows that the amount is very low, ~0.1 P_i per tau molecule even after 24 h. None of the bands show a reaction with the Alzheimer-specific antibodies (such as SMI34 or AT8 or others, not shown). (b) Top, SDS gel of htau40 (the largest human isoform) without and with phosphorylation by p34−cycA for increasing periods. Note that there is no M_r shift (the lower band is a proteolytic breakdown product). Bottom, autoradiogram of the SDS gel, showing increasing phosphorylation. However, even after 23 h there is only ≈2.6 P_i per tau molecule incorporated. As in (a) there is no reaction with the Alzheimer-specific antibodies.

MAP kinases

After these negative results we turned our attention to the family of MAP kinases. These proteins share the following general features: (i) M_r ~40−45 kDa, (ii) cross-reaction with an anti-MAP kinase antibody directed against a conserved sequence, and (iii) activation by phosphorylation at Tyr and Thr (Payne *et al.*, 1991; residues T183 and Y185 in the p42/MAPK and ERK2 sequences, see Her *et al.*, 1991; Boulton *et al.*, 1991). Thus the active enzyme can be recognized by a phosphotyrosine antibody. We first used a MAP kinase from *Xenopus* oocytes purified to homogeneity following Haccard *et al.* (1990) and Haccard *et al.* (unpublished). The enzyme incorporated ~6 P_i into tau, and it generated the same large M_r shift as the brain extract (Figure 2a). All Alzheimer-specific tau antibodies tested showed a positive reaction (Figure 2b); they include the monoclonal antibodies SMI34 (Figure 2b, lanes 3 and 4) and AT8 (lanes 5 and 6), as well as the antibodies SMI31, SMI35 and SMI310 (not shown). They all recognize Ser-Pro motifs in different states of phosphorylation (Biernat *et al.*, 1992; Lichtenberg-Kraag *et al.*, 1992). Thus, in terms of these criteria the effects of this kinase were indistinguishable from the brain extract used previously. This strongly suggested that a similar enzyme was dominant in the extract, at least with regard to tau. The purified enzyme from *Xenopus*

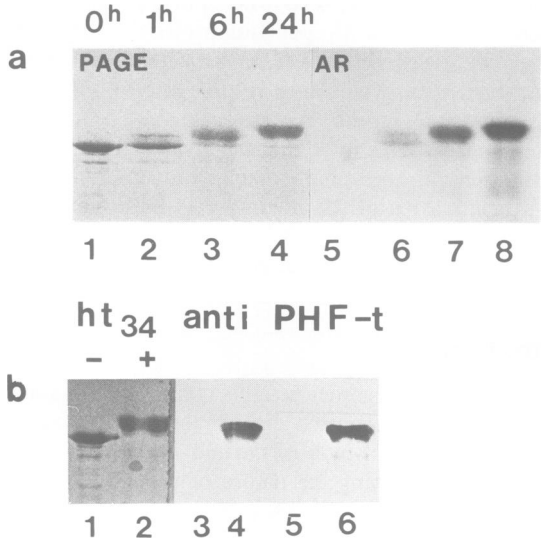

Fig. 2. Phosphorylation of bacterially expressed tau with MAP kinase from *Xenopus* oocytes (purified according to Haccard *et al.*, 1990). (**a**) Left, SDS gel of htau34 without and with phosphorylation by MAP kinase for increasing times (lanes 1−4). Right, autoradiogram (lanes 5−8). Note the M_r shift concomitant with the phosphorylation. After 24 h ~5−6 P_i per tau become incorporated. (**b**) Lanes 1 and 2, SDS gel of htau34 without and with phosphorylation by *Xenopus* oocyte MAP kinase for 24 h. Lanes 3 and 4, immunoblot with monoclonal antibody SMI34; lanes 5 and 6, immunoblot with antibody AT8. Both antibodies recognize phosphorylated epitopes specific for PHF tau (lanes 4 and 6; see Lichtenberg-Kraag *et al.*, 1992; Biernat *et al.*, 1992).

oocytes reacted with the anti-MAP kinase antibody as well as with the anti-phosphotyrosine antibody, as expected of the activated form of the enzyme (not shown, see Haccard *et al.*, 1990).

The next step was to identify the MAP kinase in the mammalian brain extract. The extract was incubated with MgATP and phosphatase inhibitors (vanadate, okadaic acid, pNPP) at 37°C and probed at different time intervals with the antibodies against phosphotyrosine and MAP kinase. Figure 3 (lanes 1–5) shows that a variety of proteins in the extract become tyrosine phosphorylated under these conditions, including a 42 kDa band which is hardly visible on SDS gels of the extract (cf. Figure 6 below, lane 1). The same band is recognized by the anti-MAP kinase antibody (Figure 3, lanes 6–10). The tyrosine phosphorylation increases with incubation time and becomes saturated after 2 h (compare time series in Figure 3, left). This confirmed that a MAP kinase was present in the brain extract.

In purifying the brain MAP kinase we basically followed the procedures developed for other sources (e.g. MAP kinase, Ray and Sturgill, 1988; MBP kinase, Ahn *et al.*, 1990; ERKs, Boulton *et al.*, 1991) with modifications as illustrated in Figures 4 and 5. The preparation consisted of five main stages: (i) preparation of a high speed supernatant brain extract, (ii) ammonium sulfate fractionation, (iii) ion exchange chromatography, (iv) gel filtration, (v) hydrophobic interaction chromatography. At each stage the activity was monitored by the phosphorylation of tau, as determined by the incorporation of radioactivity and immunoblotting with phosphorylation dependent tau antibodies. The presence of MAP kinase was demonstrated by immunoblotting with antibodies against MAP kinase and phosphotyrosine. When the extract was precipitated with ammonium sulfate, most of the activity was found in the 40% fraction. During anion exchange chromatography on Mono Q four to five peaks of kinase activity were resolved with increasing salt concentration (Figure 4A). The two major peaks (fractions around 13–15 and 16–21) were the only ones containing MAP kinase as judged by the antibody response (Figure 4B).

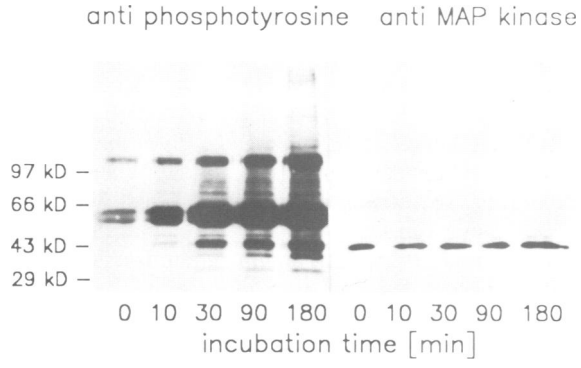

Fig. 3. Activation of MAP kinase in brain extract: the 40% ammonium sulfate fraction of porcine brain extract was desalted and incubated at 37°C with 2 mM $MgCl_2$ and 2 mM ATP, 1 μM okadaic acid and 40 mM pNPP. Samples were withdrawn at different times and electrophoresed on a 4–20% gradient SDS gel. The gel was immunoblotted with monoclonal anti-phosphotyrosine antibody Z027 and monoclonal anti-MAP kinase antibody Z033. Note that the 42 kDa protein that stains with MAP kinase antibodies (right half) becomes reactive with phosphotyrosine antibodies during the incubation, starting around 30 min (left half).

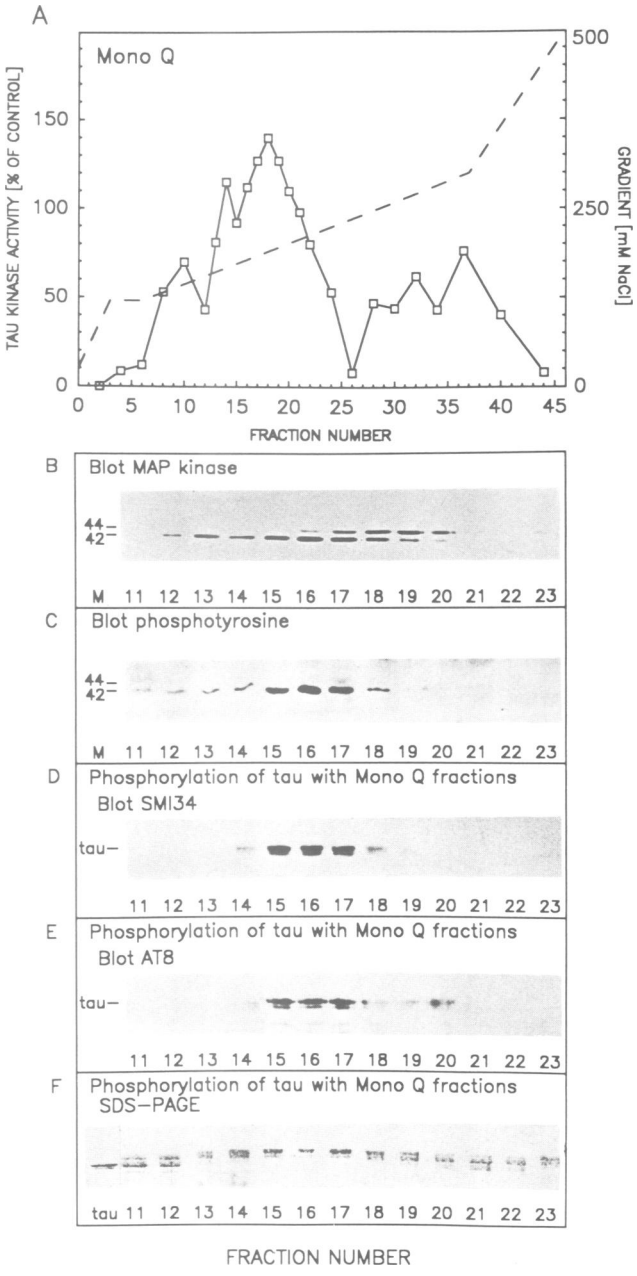

Fig. 4. Anion exchange chromatography of 250 mg protein from the kinase-rich ammonium sulfate precipitate (35–45%) of porcine brain extract, resuspended in buffer A, and run on a Mono Q HR 10/10 FPLC column. (**A**) Fractions were screened for tau kinase activity (in c.p.m. incorporated per 10 pmol of tau) and compared with the resuspended ammonium sulfate precipitate as control, taken as 100% activity. The maximum is around fraction 18. (**B** and **C**) Fractions 11–23 containing tau kinase activity were submitted to 4–20% gradient SDS–PAGE and immunoblotted with antibodies against MAP kinase (B) and tyrosine phosphate (C). Panel (B) shows that there are two isoforms of MAP kinase at 42 kDa (peaking around fraction 16) and 44 kDa (trailing behind the 42 kDa protein). Only the 42 kDa component contains phosphotyrosine (C). (**D** and **E**) Tau samples phosphorylated by fractions 11–23 were electrophoresed on 7–15% gradient gels and immunoblotted with the PHF-specific antibodies SMI34 (D) and AT8 (E), both of which depend on the phosphorylation of Ser-Pro motifs in tau. Note that the phosphotyrosine containing 42 kDa kinase phosphorylates tau in a PHF-like fashion (fractions 15–17). (**F**) 7–15% gradient PAGE of the phosphorylated tau samples. Note the prominent gel shift induced by fractions 15–17.

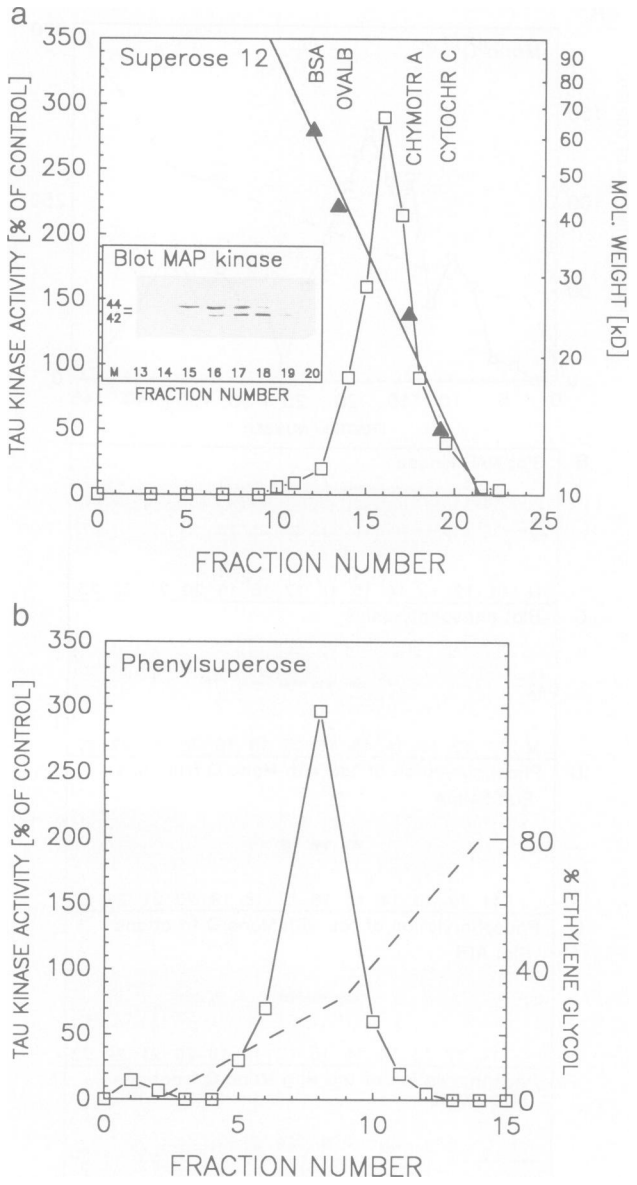

Fig. 5. Further purification of active MAP kinase fractions from Mono Q FPLC. (**a**) Gel filtration of the Mono Q pool (fractions 15–17) on a Superose 12 FPLC column equilibrated and eluted with buffer S. The elution position of marker proteins (BSA, 66 kDa; ovalbumin, 43 kDa; chymotrypsinogen A, 25 kDa; cytochrome *c*, 14 kDa) is indicated. The kinase activity elutes in a single peak with an apparent mol. wt around 30 kDa. The blot with anti-MAP kinase (insert) shows that the peak contains both isoforms at $M_r \approx 42$ and 44 kDa. (**b**) Fractions 15–18 from the Superose 12 sizing column were pooled and submitted to hydrophobic interaction chromatography on a phenyl Superose FPLC column equilibrated with buffer S containing 250 mM NaCl and eluted with a gradient of 0–80% ethylene glycol (dashed) and 250–0 mM NaCl in the same buffer. The kinase activity elutes in a single peak at around 30% ethylene glycol.

On immunoblots they contained two distinct species, with $M_r \approx 42$ and 44 kDa. The 42 kDa component eluted ahead of the 44 kDa component and contained the major part of the two proteins (~80–90%, Figure 4B). Immunoblots with anti-phosphotyrosine antibodies showed a reaction mainly with the 42 kDa band (Figure 4C).

When tau was phosphorylated with the fractions containing the 42 kDa phosphotyrosine protein it showed the same large M_r shift on SDS gels as after incubation with the brain

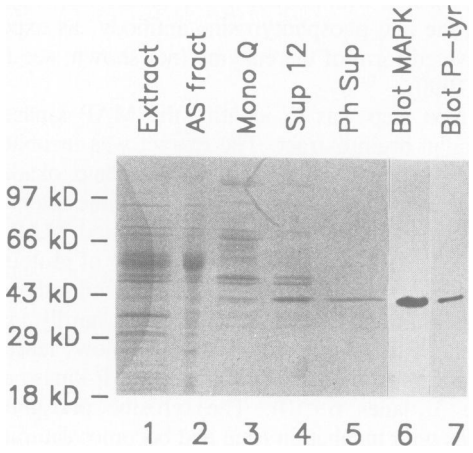

Fig. 6. SDS gel showing purification steps of brain MAP kinase. The positions of marker proteins are indicated on the left. Lane 1, crude brain extract; lane 2, 35–45% ammonium sulfate precipitate of brain extract; lane 3, pool of fractions 15–17 of Mono Q ion exchange chromatography; lane 4, fraction 16 of Superose 12 gel filtration; lane 5, pool (concentrated 10-fold) of fractions 7–9 from phenyl Superose chromatography; lanes 6 and 7, immunoblots with monoclonal antibodies against MAP kinase and phosphotyrosine, showing that the band at 42 kDa is MAP kinase and tyrosine phosphorylated.

extract or with the MAP kinase from *Xenopus* oocytes (Figure 4F). In addition the phosphorylated tau showed the antibody response with anti-PHF antibodies SMI34 (Figure 4D), AT8 (Figure 4E) or others (SMI33, SMI31, SMI310, not shown). Surprisingly, phosphorylation with the second half of the major activity peak (fractions 18–21) generated a smaller gel shift and no Alzheimer-like antibody response with SMI34 and only a faint reaction with AT8 (Figure 4D–F). This means that this fraction phosphorylates tau in a way that leaves it nearly invisible for the Alzheimer antibodies tested.

The next step was to purify the MAP kinase by gel filtration using Superose 12 FPLC (Figure 5a). Again, the fractions showing immunoreactivity with MAP kinase antibodies (nos 15–18) contained two components with the 42 kDa component trailing (Figure 5a, insert) as well as the tau kinase activity (not shown). The gel filtration yielded an apparent molecular weight ~30 kDa, rather than the expected 42–44 kDa, consistent with earlier reports (e.g. Ray and Sturgill, 1988; Ahn *et al.*, 1990) and indicating some interaction with the Superose matrix. When the peak fraction (no. 16) was concentrated 10-fold its specific phosphorylating capacity became much higher than that of the crude extract or the ammonium sulfate fraction. About 14–16 phosphate residues were incorporated into the tau isoform htau34 (specific activity 7.1 nmol/min/mg). In the final purification step (hydrophobic interaction FPLC using phenyl Superose, Figure 5b) the kinase was purified to apparent homogeneity. The SDS gel of Figure 6 illustrates the various stages of purification. The last step (lane 5) contains only the single 42 kDa component and reacts with the antibodies against MAP kinase (lane 6) and phosphotyrosine (lane 7). This purified fraction also incorporates 14–16 P_i into tau. By comparison, the enzyme incorporates ≈34–37 P_i into MAP2 from porcine brain.

Figure 7 shows the time course of the phosphorylation of tau with the final MAP kinase preparation, documented by the gel shift in SDS–PAGE, autoradiography and

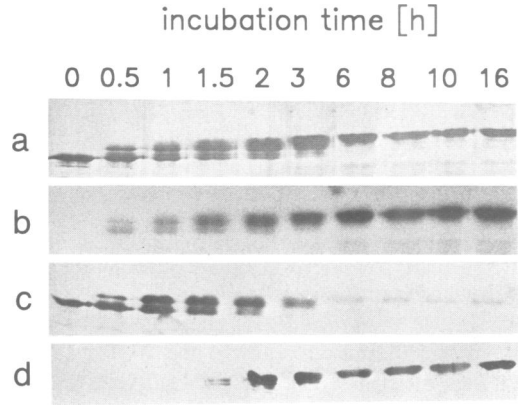

Fig. 7. Time course of phosphorylation of recombinant human tau 34 with the MAP kinase purified from brain. (**a**) Silver stained SDS−PAGE of htau34 after incubation with the kinase between 0 and 16 h. While the unphosphorylated tau is a single band (t = 0), phosphorylation leads to a shift to higher M_r with more or less well defined intermediate stages, suggesting that the phosphorylation occurs in successive steps. (**b**) Autoradiography of the same gel. The phosphate incorporated is as follows: 1 h, ≈2 P_i; 2 h, ≈6 P_i; 6 h, ≈9.5 P_i; 16 h, ≈12 P_i. (**c**) Immunoblot with the antibody TAU1 whose reactivity is lost after 2−3 h (following the phosphorylation of S199 and S202, see Lichtenberg-Kraag *et al.*, 1992). (**d**) Immunoblot with the PHF-specific antibody AT8 whose reactivity requires the phosphorylation of S199 and S202.

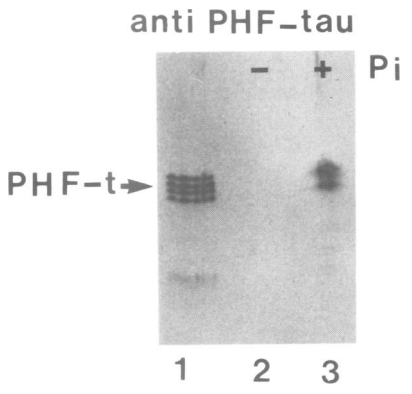

Fig. 8. Lane 1, tau protein from PHFs of Alzheimer brain, immunoblotted with the PHF-specific antibody AT8; lane 2, PHF tau after dephosphorylation with alkaline phosphatase; lane 3, after rephosphorylation with brain MAP kinase (fraction 16 of Superose 12 column, see Figure 5A). The PHF-like antibody response is phosphorylation dependent and restored by MAP kinase.

antibody response. It is remarkably similar to the results obtained with the brain extract (Lichtenberg-Kraag *et al.*, 1992). In particular, the phosphorylation can be broadly subdivided into three stages, with the first containing the most conspicuous gel shift (lanes 1−4). However, the transformation of tau to the Alzheimer-like state occurs only during the second stage (lanes 5 and 6), as judged from the disappearance of the TAU1 reactivity and the emergence of the AT8 response, both of which are antibodies sensitive to the phosphorylation state of serine pair S199/S202 in a complementary fashion (for details see Biernat *et al.*, 1992). The same result is achieved with the other PHF-specific antibodies SMI31, SMI34, SMI35, SMI310 (data not shown). The specific activity of the highly purified kinase was ~45 nmol/min/mg, using htau34 as substrate, or 155 nmol/min/mg for MAP2. These values correspond to the V_{max} with regard to the substrate ATP; the K_M value for ATP was 170 μM. For comparison, the brain extract showed total kinase activities of 0.3 and 0.5 nmol/min/mg for htau34 and MAP2, respectively. Thus the activity is enriched at least 150-fold during purification.

When ATP is omitted in the purification scheme the MAP kinase becomes inactive in phosphorylating tau at later stages of purification, and there is no detectable tyrosine phosphorylation of the enzyme either. In analogy with the other MAP kinases this suggests that the extract contains a protein tyrosine kinase or other factor(s) capable of activating MAP kinase by phosphorylation; once the activation step has occurred, MAP kinase can phosphorylate tau, even when the tyrosine kinase is lost during the subsequent purification steps. Similarly, on immunoblots the enzyme showed no detectable autophosphorylation at tyrosine leading to autoactivation (such a process was reported by Seger *et al.*, 1991, and Wu *et al.*, 1991, although it appears to be of secondary importance compared to physiological activation). By quantification of MAP kinase bands from immunoblots of a brain extract sample activated in the presence of [γ-^{32}P]ATP (200 Ci/mmol) we estimate that the concentration of the phosphorylated enzyme is on the order of 1 μg per gram of brain tissue extracted.

MAP kinase and tau in Alzheimer brain

Thus far our arguments relating MAP kinase to tau were based on the phosphorylation of recombinant human tau and its reaction with Alzheimer-specific antibodies whose epitopes and phosphorylation dependence were determined independently. However, the effect of MAP kinase can also be demonstrated directly with PHF tau isolated from Alzheimer brain (Figure 8). This tau protein was resolved into four bands, all of which stain with the PHF-specific antibody AT8 (lane 1). The antibody response is lost upon dephosphorylation of tau with alkaline phosphatase (lane 2), but it re-emerges when the dephosphorylated protein is rephosphorylated with the purified MAP kinase from brain (lane 3). Similar results are obtained with PHF-specific antibodies directed against other epitopes (not shown, see Lichtenberg-Kraag *et al.*, 1992).

We also found MAP kinase in human brains. A comparison was made between brain extracts from four normal humans and four Alzheimer patients, all of whom were aged 60−90 years and autopsied 30−140 min post mortem. The enzyme was activated with ATP and capable of phosphorylating tau; its presence was demonstrated with antibodies against MAP kinase and phosphotyrosine (details elsewhere).

Discussion

In our previous studies we had shown that mammalian brain extract contains a protein kinase which is capable of transforming tau protein into the PHF-like state, as judged by several criteria such as gel shift, antibody response and phosphorylation sites (Biernat *et al.*, 1992; Lichtenberg-Kraag *et al.*, 1992). The phosphorylation was mainly on Ser/Thr-Pro motifs, suggesting that the activity of the brain extract with regard to tau might be dominated by a proline directed Ser/Thr kinase. In this study we identify the kinase as a MAP kinase; we describe the purification of the active

enzyme to homogeneity from brain; and we show that the pure kinase can transform tau into the Alzheimer-like state, as judged by the gel shift, phosphorylation of Ser-Pro motifs and response to PHF-specific antibodies. We also show that other proline directed kinases such as p34(cdc2) complexed with cyclin A or B do not have this effect.

What are the criteria for calling our enzyme a MAP kinase? (i) The protein is recognized by a monoclonal anti-MAP kinase antibody raised against a peptide conserved in all known MAP kinase sequences and cross-reacting with all of them (see Zymed product information). (ii) The enzyme is activated by tyrosine phosphorylation, like all other MAP kinases, and the active form is recognized by an anti-phosphotyrosine antibody (Ray and Sturgill, 1988; Boulton and Cobb, 1991). (iii) The protein has an M_r of 42 kDa on SDS gels, but only $\approx$30 kDa by gel filtration (compare Ray and Sturgill, 1988; Ahn *et al.*, 1991). (iv) The brain kinase (as well as the one from *Xenopus* oocytes) has the required substrate specificity (Gonzalez *et al.*, 1991; Lewis *et al.*, 1991).

A variety of names are currently in use for members of the MAP kinase family (for reviews see Cobb *et al.*, 1991; Sturgill and Wu, 1991; Thomas, 1992). The original MAP kinase from 3T3-L1 cells is now $p42^{mapk}$ (Her *et al.*, 1991). It is similar to the enzyme ERK2 cloned from a rat brain cDNA library (Boulton *et al.*, 1991), to the MBP kinase E3 from Swiss 3T3 cells (Ahn *et al.*, 1990) and to the *Xenopus* MAP kinase-1 (Gotoh *et al.*, 1991). A common feature is that the kinases usually come in (at least) two isoforms with M_r values around 42 and 44 kDa. The larger one is also named ERK1 (Boulton *et al.*, 1991) or MBP kinase E4 (Ahn *et al.*, 1990). Our active enzyme probably corresponds to ERK2, based on the following criteria: (i) It is the smaller of the two MAP kinase isoforms, with M_r = 42 kDa; (ii) it elutes somewhat earlier on the Mono Q column (cf. Gomez *et al.*, 1990; Boulton and Cobb, 1991); (iii) it is more abundant than the 44 kDa component, similar to ERK2 in rat brain (Boulton *et al.*, 1991). However, for a more detailed comparison we will have to await the sequence of the protein (experiments in progress).

It is interesting to note that the MAP kinase was originally named MAP2 kinase because the microtubule associated protein MAP2 was a good substrate *in vitro* (for review see Sturgill and Wu, 1991). Changing to MAP kinase (mitogen activated protein kinase) was intended to avoid the name of a substrate that might not have a physiological relevance. It now turns out that tau, a protein closely related to MAP2 (Lee *et al.*, 1988; Lewis *et al.*, 1988), may be an important substrate of the kinase, giving again some justification for the original name.

Several authors have proposed consensus sequences for MAP kinase derived from peptide substrates. The minimal one is Ser/Thr-Pro, a more extended one is Pro-Xaa-Ser/Thr-Pro, with Xaa preferably neutral or basic (Clark-Lewis *et al.*, 1991; Gonzalez *et al.*, 1991; Lewis *et al.*, 1991). Human tau has four of these extended motifs, $PKT^{181}P$, $PKS^{235}P$ and two nested ones in the sequence PGS^{202}-$PGT^{205}P$. On one hand we know that there are more phosphorylation sites on tau than just these motifs, suggesting that 'consensus' should be taken with a grain of salt; but on the other hand it is interesting that all of the consensus sequences are phosphorylated by the brain extract, as determined from tryptic phosphopeptides and antibody labelling (Biernat *et al.*, 1992; Lichtenberg-Kraag *et al.*, 1992, and unpublished data).

A notable difference between the kinase activities in brain extract and after purification is that the pure enzyme incorporates twice or more phosphates into tau, $\approx 14-16$ per molecule. This suggests that not only Ser-Pro but also most of the Thr-Pro motifs become phosphorylated (so far all Ser-Pro motifs, and two of the Thr-Pro have been identified directly by phosphopeptide sequencing of htau40 phosphorylated with brain extract of 24 h, for sequencing methods see Meyer *et al.*, 1991). There are six to seven Ser-Pro motifs (depending on isoform) and 10 Thr-Pro motifs, giving a total of $16-17$ potential phosphorylation sites. This is in qualitative agreement with the observed level of phosphorylation.

If the MAP kinase has a physiological role in tau phosphorylation one might expect it to occur in the same cell compartments. Tau is thought to be mainly in axons, judging from the reactivity with TAU1, although dephosphorylation reveals that it is also present in the nerve cell body and dendrites (Binder *et al.*, 1985; Papasozomenos and Binder, 1987). Since we know now that the reactivity of TAU1 disappears upon phosphorylation of S199 and S202, and since these serines are phosphorylated by MAP kinase (inducing the AT8 immune response), it is possible that the kinase occurs mainly in the somatodendritic compartment. This would be consistent with the observation that the neurofibrillary tangles occur primarily in this compartment as well (see Braak and Braak, 1991), implying a loss of MAP kinase regulation. We would also expect that the phosphorylation of tau by MAP kinase enhances the assembly of tau into PHFs *in vivo* and possibly *in vitro*. These relationships will have to be verified by future experiments.

The regulation would presumably involve the phosphorylation of T183 and Y185 of p42/MAP kinase (Anderson *et al.*, 1990; Payne *et al.*, 1991; Boulton *et al.*, 1991), but there may be even more phosphorylation sites on the kinase. The kinase is activated by mitogens and growth factors; however, the receptor tyrosine kinases probably do not affect MAP kinase directly so that the regulatory cascade is more complex (Ahn *et al.*, 1991; Gomez and Cohen, 1991). In this study we have used an antibody against phosphotyrosine to monitor the state of phosphorylation of MAP kinase. We find that the enzymes from frog oocytes as well as from mammalian brain phosphorylate tau only when they are tyrosine phosphorylated.

We finally note that certain sites of tau can be phosphorylated by other kinases. Examples are CaM kinase (phosphorylating S416, Steiner *et al.*, 1990), or PKA and PKC (Baudier and Cole, 1987; Steiner *et al.*, 1990), but these are not proline directed kinases. Several other proline directed Ser/Thr kinases related to the cytoskeleton have been reported in the literature, e.g. neurofilament kinases (Wible *et al.*, 1989; Roder and Ingram, 1991) and a tubulin-dependent kinase (Ishiguro *et al.*, 1991). Of particular interest is the relationship with neurofilament-associated kinases since there are common motifs with tau of the Lys-Ser-Pro-Val type (residues 395–398 in htau40). Indeed, such motifs appear to be phosphorylated in the Alzheimer A68 protein (Lee *et al.*, 1991), and a neurofilament-associated kinase phosphorylates this motif both in neuro-

filament proteins and in tau (Roder and Ingram, 1991). The relationship and distribution of these kinases in brain remain to be elucidated.

In summary, the significance of this work is that we have now purified and identified a kinase that is likely to be a key factor in the transformation of tau from the normal to the Alzheimer state. This can be demonstrated with normal tau isolated from human or other mammalian brains, with recombinant tau expressed in *Escherichia coli*, or with tau from Alzheimer PHFs. The kinase is a member of the MAP kinase family, probably $p42^{mapk}$ or ERK2, it phosphorylates Ser-Pro or Thr-Pro motifs in tau, and it is activated by tyrosine phosphorylation. The kinase is present in mammalian brain, particularly in normal human brain as well as brain from Alzheimer patients. In brain extracts the kinase is the dominant one with regard to tau phosphorylation. These data will help dissect the cascade of events that leads to the abnormal phosphorylation of tau protein and its deposition into paired helical filaments. This in turn will hopefully shed some light on the molecular causes of Alzheimer's disease.

Materials and methods

Tau proteins

The human tau cDNA clones were kindly provided by M.Goedert (see Goedert *et al.*, 1989) and were expressed in the expression vector pNG2, a derivative of pET-3b (Studier *et al.*, 1990), modified by removal of *PstI*, *Hin*dIII, *Nhe*I and *Eco*RV restriction sites for convenient engineering. For the expression we used the BL21 (DE3) *E.coli* system (Studier *et al.*, 1990). The numbering of residues used here refers to the sequence of htau40, the largest of the human isoforms (441 residues). For the isolation of the constructs we made use of the heat stability of the protein; they were separated by FPLC Mono S (Pharmacia) chromatography (for details see Hagestedt *et al.*, 1989). Tau from bovine brain was prepared as described (Hagestedt *et al.*, 1989), again making use of heat stability and Mono S chromatography. Tau from paired helical filaments (PHF) was prepared according to Greenberg and Davies (1990).

Monoclonal antibodies

The antibody AT8 reacting specifically with tau from Alzheimer PHFs was obtained from Innogenetics S.A. (Gent, Belgium, see Biernat *et al.*, 1992; Mercken *et al.*, 1992). The antibody SMI34 was obtained from Sternberger Monoclonals, Inc. (Baltimore, MD, USA; see Sternberger *et al.*, 1985). The antibody TAU1 was a generous gift of Dr L.Binder (Binder *et al.*, 1985). Monoclonal anti-phosphotyrosine antibody Z027 and monoclonal anti-MAP kinase antibody Z033 were from Zymed Labs, South San Francisco, CA, USA).

Phosphorylation assays

1–4 μl of kinase fractions were added to aliquots of a solution of htau34 (0.25 mg/ml) or MAP2 (0.1 mg/ml) in 40 mM HEPES pH 7.4 containing 2 mM [γ-^{32}P]ATP (10–20 Ci/mmol), 3 mM $MgCl_2$, 2 mM DTT, 5 mM EGTA, 5 μM okadaic acid and 0.2 mM PMSF, to a final volume of 20 μl. After incubation at 37°C for different time intervals, the reaction was stopped by the addition of electrophoresis sample buffer. After electrophoresis on 7–15% gradient gels the tau bands were stained with Coomassie Blue, cut out and the incorporated radioactivity was determined by Cerenkov counting. In some cases phosphorylated tau samples were blotted with antibodies AT8, SMI34 or TAU1.

Purification of the protein kinase

Fresh porcine brains obtained from a local slaughterhouse were cleaned from meninges and blood vessels. 250 g of tissue was homogenized at 4°C in 150 ml of buffer A [25 mM Tris–HCl pH 7.4 containing 25 mM NaCl, 2 mM EGTA, 2 mM DTT, phosphatase inhibitors [1 μM okadaic acid (LC Services, Woburn, MA, USA)], 40 mM pNPP (paranitrophenyl phosphate; Fluka, Buchs, Switzerland), 1 mM Na_3VO_4 and protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, pepstatin and aprotinin) and centrifuged at 100 000 *g* for 30 min. The supernatant was submitted to ammonium sulfate fractionation. The precipitate of 40% or between 35 and 45% was collected by centrifugation, homogenized in 20 ml of buffer A and quickly desalted over a battery of PD10 columns (Pharmacia) equilibrated with buffer A. The protein fractions were collected, clarified by ultracentrifugation, diluted 5- to 10-fold with buffer A and incubated for 3 h at 37°C after the addition of 2 mM MgATP ('kinase activation step'). After addition of 40% (v/v) ethylene glycol, the solution could be stored at −20°C with very little loss of activity. This mixture containing ~250 mg total protein was loaded on a Mono Q HR10/10 FPLC column (Pharmacia Fine Chemicals) operated at 4°C and equilibrated with 25 mM Tris–HCl pH 7.4, 25 mM NaCl, 10 mM pNPP, 2 mM EGTA and 0.5 mM DTT. Elution was performed with a gradient of 25–500 mM NaCl in the same buffer over 80 min with a flow rate of 2 ml/min and 4 ml fractions were collected. Fractions were assayed for their phosphorylating activity with recombinant htau34 as substrate and by Western blotting with anti-MAP kinase and anti-phosphotyrosine antibodies. Active fractions were pooled and concentrated by centifugation through Centriprep-10 microconcentrators (Amicon) to a final volume of 1–2 ml. Portions of 200 μl were submitted to gel filtration on a Superose 12 HR 30/10 column (Pharmacia) equilibrated and eluted with buffer S (25 mM Tris–HCl pH 7.4 containing 2 mM EGTA, 0.5 mM DTT, 10 mM pNPP, 5 mM ATP, 5 mM $MgCl_2$ and 0.5 mM PMSF) at a flow rate of 0.3 ml/min and fractions of 0.6 ml were collected. Active fractions from this column were pooled and submitted to hydrophobic interaction chromatography on a phenyl Superose HR 5/5 column (Pharmacia) equilibrated with buffer S containing 250 mM NaCl and eluted with a gradient of 0–80% ethylene glycol and 250–0 mM NaCl in the same buffer. The flow rate started at 0.3 ml/min and was stepwise lowered to 0.1 ml/min and fractions of 1.5 ml were collected. Active fractions were pooled, concentrated 50-fold and stored frozen in liquid nitrogen.

Miscellaneous

SDS–PAGE was conducted on 4–20% or 7–15% gradient gels. Immunoblotting was done on Immobilon membranes (Millipore); non-specific binding was blocked by 1% bovine serum albumin. The bound antibody was detected by a peroxidase conjugated second antibody (anti-mouse IgG, Dakopatts), using a chemiluminescence detection system (Amersham).

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